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Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice

(skin carcinoma)

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ABSTRACT **Certain "high-risk" anogenital human papillomaviruses (HPVs) have been associated with the majority of human cervical carcinomas. In these cancers, two papillomaviral genes, E6 and E7, are commonly expressed. In this study we provide evidence that expression of the E6 and E7 genes from the high-risk HPV-16 in the skin of transgenic mice potentiated the development of preneoplastic lesions, and a high percentage of these epidermal lesions subsequently developed into locally invasive cancers. High levels of E6/E7 expression were found in these tumors relative to the preneoplastic lesions, and expression was localized to the proliferating, poorly differentiated epidermal cells. Also, the p53 and Rb genes were found to be intact, not mutationally inactivated, in representative skin tumors. These findings demonstrate that the E6 and E7 genes from a papillomavirus etiologically associated with human cervical cancer can contribute to the development of epidermal cancers in an animal model.**

Human papillomaviruses (HPVs) are small DNA viruses that infect various epithelial tissues, including the skin epidermis and epithelial lining of the anogenital tract. A subset of anogenital papillomaviruses that includes the HPV-16, -18, -31, and -33 genotypes is found to be associated with cervical carcinomas. The selective expression of the papillomavirus E6 and E7 genes in these HPV-positive cervical lesions suggests that these viral genes may contribute to the initiation and/or tumorigenic progression of the disease. In tissue culture cells, although E6 and E7 exhibit an immortalizing activity (1–3), additional oncogenic activities are necessary for tumorigenicity (4). The E7 protein has been demonstrated to associate with the retinoblastoma tumor suppressor gene product (5) and this association alters Rb protein activities, including its capacity to complex with the cellular transcription factor E2F (6). The E6 protein associates with the p53 tumor suppressor gene product (7) and this association leads to the increased instability of p53 protein (8). This functional inactivation of the Rb and p53 proteins by the HPV E7 and E6 proteins, respectively, has been argued to obviate the need for mutational inactivation in the primary HPV-positive cervical cancers (9, 10).

To address the role of viral genes in HPV-associated carcinogenesis *in vivo*, we have generated an animal model in transgenic mice to monitor the activities of the HPV-16 E6 and E7 oncogenes (11). The E6 and E7 genes of HPV-16, the papillomavirus genotype most commonly associated with cervical carcinoma, were placed under the transcriptional control of the αA crystallin promoter, targeting expression of the transgenes to the mouse ocular lens (11). Three αAHPV16E6/E7 germ-line transgenic founders were generated; all three lines of mice expressed the transgenes in the lens and exhibited bilateral microphthalmia with 100% penetrance. This phenotype resulted from the impairment of lens fiber cell differentiation and induction of lens epithelial cell proliferation. Adult mice in the line expressing highest levels of E6 and E7 genes developed lens tumors. We now report that ectopic expression of the E6 and E7 transgenes in the skin of these αAHPV16E6/E7 transgenic mice correlates with a high incidence of preneoplastic skin lesions and the subsequent development of carcinomas.

MATERIALS AND METHODS

Histological Analysis of Skin Samples. Tissue samples were fixed in 4% paraformaldehyde overnight at 4°C, transferred to phosphate-buffered saline, and embedded in paraffin. Serial sections, 5 μm in thickness, were stained with hematoxylin/eosin or used for immunostaining or *in situ* hybridization. Immunostaining was performed using a 1:20 dilution of anti-human proliferating cell nuclear antigen (PCNA) monoclonal primary antibody (Boehringer Mannheim) and fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG secondary antibody.

***In Situ* Hybridization.** *In situ* hybridizations were performed as described (12). The cRNA probes for type I epidermal-specific transglutaminase and α1 type IV collagen were synthesized *in vitro* from the template DNAs, pTG13 (13) and pCIV 1225 (14), respectively, using UTP[α-^{35}S]. The E6 and E7 open reading frames from HPV-16 were independently cloned into pGEM3Z and each was used to generate cRNA probe, incorporating UTP[α-^{35}S] and CTP[α-^{35}S], and then combined for hybridization. Slides were exposed for 7 days at 4°C in the dark before developing.

Single-Strand Conformation Polymorphism (SSCP) Analysis. For SSCP analysis, RNA/PCR products were generated using [α-^{32}P]dATP during amplification, and products were resolved by gel electrophoresis using 0.5× MDE gel mix with 0.5× Tris/borate/EDTA buffer and 10% glycerol (AT Biochem, Malvern, PA).

RESULTS

Incidence of Abnormal Skin and Skin Tumors in αAHPV16E6/E7 Transgenic Mice. In characterizing the patterns of transgene expression in tissues from neonatal αAHPV16E6/E7 transgenic mice, we noted expression of E6 and E7 in several nonlenticular tissues, particularly in one line of mice (11). Upon breeding this line of mice, line 19, we noted the frequent appearance of abnormal-looking skin, appearing at ≈3 months of age. The incidence of abnormal skin was higher and appearance was earlier in mice homozygous for the transgene than in hemizygous mice (Table 1, 48%

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Abbreviations: HPV, human papillomavirus; SSCP, single-strand conformation polymorphism; PCNA, proliferating cell nuclear antigen; FITC, fluorescein isothiocyanate.

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Table 1. Incidence of skin abnormalities in αAHPV-16E6/E7 mice

	Age of onset, months					
	<3		3–6		>6	
Skin condition	h	H	h	H	h	H
Abnormal skin*	0/107	3/127	2/107	15/127	13/107	61/127
Carcinoma†	0/3		0/17		15/74	

A total of 107 hemizygotes and 127 homozygotes from line 19 was used in the study. h, Mice hemizygous for transgenic allele; H, mice homozygous for transgenic allele.
*Number of mice displaying abnormal skin/total number of mice in group.
†Number of mice with abnormal skin that have developed carcinomas/total number of mice with abnormal skin.

vs. 13%). Male and female line 19 mice were affected; no abnormal skin phenotype was seen on nontransgenic mice. The abnormal skin phenotype originally presented itself as a localized area or areas of hair loss and progressed further to become dry and scaly in appearance, with up to 50% of an animal's skin becoming affected. Hair loss was found at many sites on the body and head of the line 19 mice.

Greater than 20% of the line 19 animals with abnormal skin developed squamous cell carcinomas (grades 1–3) at sites of abnormal skin (Table 1). Carcinomas appeared several months after the onset of the abnormal skin and grew quite large (0.5 cm–3 cm in diameter). At low incidence (2 of 40 skin tumors examined histologically to date), dermal fibrosarcomas developed in addition to, or instead of, carcinomas. We also noted a low incidence of skin tumors in a second line of αAHPV16E6/E7 mice, line 4 (data not shown). In addition to cancers developing at sites of abnormal skin lesions on the body, a large number of tumors (primarily carcinomas, one fibrosarcomas identified to date) >1 cm in size developed on the ears of the line 19 transgenic mice. These tumors appeared at the sites where identification tags had been placed on the mouse ear at the time of weaning. This indicates that wounding may be a cofactor in the development of these tumors. We were able to recapitulate experimentally the association between tumor incidence and wounding. Surgical incisions were made on the backs of weanlings and metal suture clips were used to close the wounds; abnormal skin and/or carcinomas developed at the sites of wounding in greater than a third (6 of 16) of the line 19 animals after 3 months, but no lesions were found at wounding sites on control, nontransgenic mice (data not shown). Preneoplastic and neoplastic lesions have also developed at sites of wounding caused by fighting among cage mates, including tumors on the male genitalia. Thus, wounding appears to be a cofactor for tumor development in these mice. The cancers that developed on the ears or skin of these mice were locally invasive but not metastatic at the time the mice were sacrificed.

Histopathology of Abnormal Skin and Skin Tumors. The skin of these transgenic mice was examined histologically to determine the morphological basis for the observed disease. The epidermis of a normal mouse is thin, consisting of one basal cell layer and one or two layers of differentiated cells, and the dermis contains numerous hair follicles (Fig. 1*A*). In contrast, the epidermis of the abnormal skin is highly thickened, with evidence of acanthosis and hyperkeratosis (Fig. 1*B*). There are far fewer hair follicles and those present appear abnormally thick. The dermis also appears thickened and is infiltrated by neutrophils, evidence of inflammation within the tissue (see Fig. 3 *A* and *D* for higher magnification views of normal and abnormal skin). In the skin tumors, the epidermis is also highly thickened (Fig. 1 *C* and *D*). In addition, epidermal cells are seen throughout the dermis, indicating that these cells are invasive (Fig. 1*D*). This latter

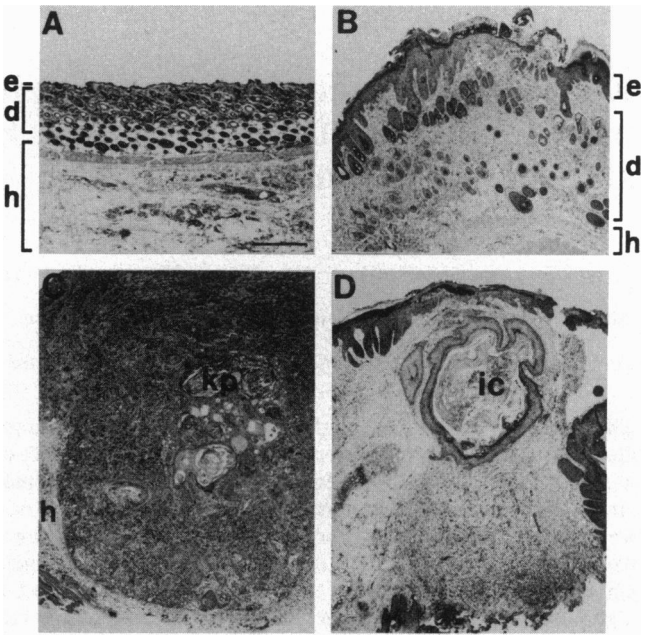

FIG. 1. Histopathology of line 19 αAHPV-16E6/E7 transgenic skin. Shown are representative sections from normal mouse back skin (*A*), back skin from a 10-month-old hemizygous transgenic mouse with abnormal skin (*B*), a grade 2 squamous cell carcinoma found on the shoulder of a 10-month-old transgenic homozygote (*C*), and a grade 3 squamous cell carcinoma found on the ear of a 14-month-old transgenic hemizygote (mouse 554) (*D*). Only a portion of the section of the tumor in *C* is shown. (Bar = 44 μm.) d, Dermis; e, epidermis; h, hypodermis; ic, inclusion cyst; kp, keratin pearl.

feature is noticeable in the grade 2 tumor (Fig. 1*C*; see Figs. 3*G* and 4*I*), where keratinocytes have penetrated completely through the dermis to the boundary with the underlying hypodermis, as well as in the grade 3 tumor, where a large, dense mass of poorly differentiated epidermal cells is localized deep in the dermis (Fig. 1*D*; see Fig. 4*A*). Also evident in these tumors are numerous keratin pearls and epidermal inclusion cysts (see Figs. 3*G* and 4 *A*, *E*, and *I* for higher magnification views).

Expression of HPV-16 E6 and E7 Genes. To determine if E6 and/or E7 expression is correlated with the skin pathology seen in the transgenic mice, RNAs isolated from different tissues were subjected to qualitative RNA/PCR analysis. Three amplification products would be predicted based upon differential splicing: a 523-bp product derived from the unspliced E6 specific transcript and 348-bp and 233-bp products derived from the E6*^E7 and E6**^E7 spliced transcripts (see figure 2 in ref. 11). The Southern blot in Fig. 2 indicates that all three predicted amplification products were detected in RNA samples from transgenic skin but not in RNA samples from nontransgenic skin. As has been demonstrated in HPV-16-positive human cervical neoplasms (15), the E6*^E7 transcript was the most abundant transcript in the transgenic skin tissue, whereas the level of the E6**^E7 transcript was low. These transcripts originated from the αA crystallin promoter as RNA/PCR using a 5′ primer positioned directly upstream of the promoter start site failed to amplify any RNA (M.J. and A.E.G., data not shown). E6 and E7 mRNAs were also detected in the skin of line 4 mice (data not shown). Thus, E6- and E7-specific transcripts are present in normal, abnormal, and tumorigenic skin samples of these transgenic mice.

Patterns of E6/E7 Expression in Abnormal Skin and Carcinomas. The pattern of E6/E7 expression in nontransgenic and abnormal skin was compared to that in skin tumors by *in situ* hybridization experiments using a probe that will detect E6-specific and E7-specific mRNA species. Low-level ex-

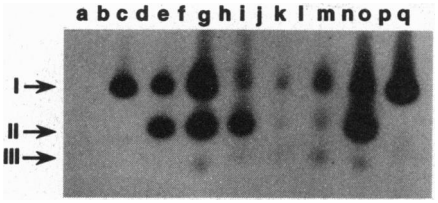

FIG. 2. Expression of E6 and E7 in line 19 transgenic mouse skins by RNA/PCR analysis: Southern blot hybridization of RNA/PCR analysis using oligonucleotides αP1 and 2 (11) as primers and [α-^{32}P]dCTP-labeled HPV-16 E6/E7 DNA as hybridization probe. The RNA samples were derived from nontransgenic FVB/N skin (lanes a–c), neonatal transgenic skin (lanes d and e), adult transgenic skin from mouse exhibiting no abnormal skin (lanes f and g), adult transgenic skin from an unaffected region of a mouse with abnormal skin (lanes h and i), adult transgenic abnormal skin (lanes j and k), skin tumor from mouse 554 (lanes l and m), and skin tumor from mouse 1706 (lanes n and o). Lanes p and q are PCR controls that contained no DNA (lane p) and 10 ng of plasmid pαAHPV-16E6/E7 (lane q) (11). The RNA samples in lanes a, d, f, h, j, l, and n were pretreated with RNase A before processing through the RNA/PCR protocol. The nontransgenic FVB/N RNA in lane c was spiked with 1×10^{10} molecules of *in vitro* transcribed E6 RNA before processing. I, 523-bp fragment (E6); II, 348-bp fragment (E6*^E7); III, 233-bp fragment (E6**^E7). The 233-bp fragment, undetectable in lane e, was detected in other neonatal transgenic skin samples analyzed (data not shown).

pression of E6/E7 was found in epidermis and dermis of abnormal skin (Fig. 3*E*). Expression of E6/E7 in the skin carcinoma (Fig. 3*H*) was qualitatively higher than in abnormal skin and appeared localized to specific regions.

To identify which cell type supports a high level of E6/E7 expression in skin carcinomas, we compared the patterns of E6/E7 expression by *in situ* hybridization to that of two genes expressed differentially within the epidermis, α1-type IV collagen and type I transglutaminase. Type IV collagen is specifically expressed in basal epidermal cells, whereas transglutaminase is expressed in differentiating epidermal cells (13, 14). For two different carcinomas, neighboring sections were hybridized to ^{35}S-labeled complementary-strand RNA probes specific for type I transglutaminase, type IV collagen, or HPV-16 E6/E7. Hybridization to the transglutaminase (Fig. 4 *B*, *F*, and *J*) and type IV collagen (Fig. 4 *C*, *G*, and *K*) probes was seen not only in the epidermis proper but also in patches within the underlying dermal layer, indicative of local invasion. Intradermal hybridization was seen in inclusion cysts and keratin pearls, and appropriate layers of these differentiating epidermal structures hybridized to either the transglutaminase or the type IV collagen probe. Strong hybridization to the type IV collagen probe in the area beneath the large inclusion cyst (Fig. 4 *B* and *C*) indicates that this dermal region is infiltrated by undifferentiated epidermal cells.

The E6/E7 probe hybridized strongly to several regions throughout the tumors (Fig. 4 *D*, *H*, and *L*). Cells that expressed E6/E7 at high levels corresponded to epidermal cells including those in the epidermis proper (Fig. 4*H*), the inclusion cysts and keratin pearls (Fig. 4 *H* and *L*), and the cells in the region below the large inclusion cyst (Fig. 4*D*) within the dermis. Comparison of the hybridization patterns of the transglutaminase, type IV collagen, and HPV-16 E6/E7 probes in these tumor sections indicates that expression of E6/E7 most closely overlaps with expression of type IV collagen. This congruence indicates that E6 and E7 are highly expressed in poorly differentiated epidermal cells in these carcinomas. Similar results were seen in other carci-

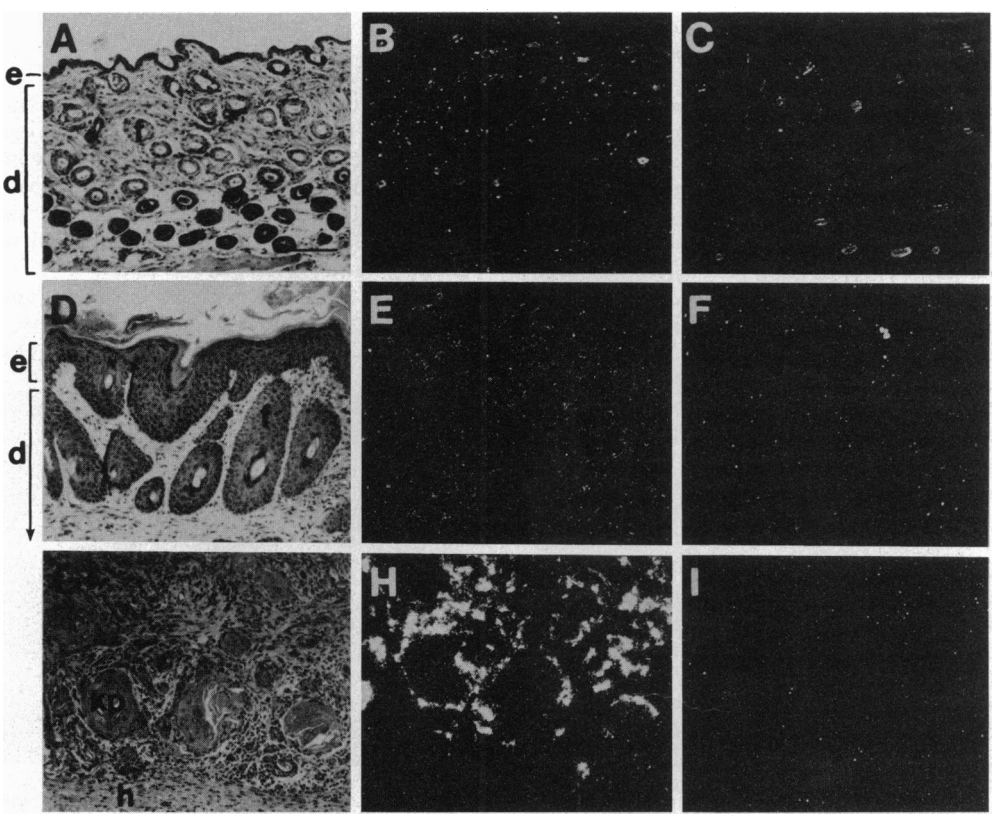

FIG. 3. Expression of E6/E7 in adult transgenic skin samples by *in situ* hybridization. Neighboring sections were taken from normal skin (*A–C*), abnormal skin (*D–F*), and a grade 2 skin carcinoma (*G–I*). (*A*, *D*, and *G*) Micrographs of sections stained with hematoxylin/eosin. (*B*, *E*, and *H*) Dark-field micrographs of sections hybridized to UTP[α-^{35}S]/CTP[α-^{35}S]-labeled HPV-16 E6/E7 antisense strand probe. (*C*, *F*, and *I*) Dark-field micrographs of sections hybridized to radiolabeled HPV-16 E6/E7 sense strand probe. The hair follicles (*B* and *C*) are refractile in dark-field optics. (Bar = 11 μm.) d, Dermis; e, epidermis; f, hair follicle; h, hypodermis; kp, keratin pearl.

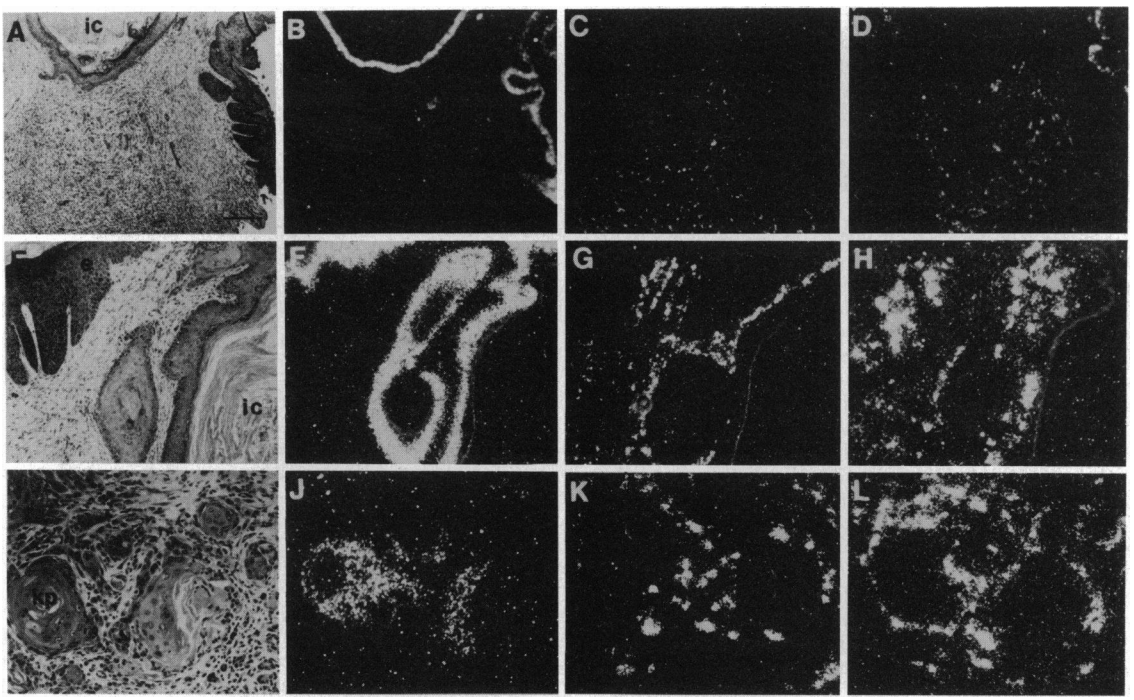

FIG. 4. Localization of E6/E7 expression in squamous cell carcinomas by *in situ* hybridization. The bright-field micrographs show at higher magnification the hematoxylin/eosin-stained sections from the grade 3 carcinoma in Fig. 1*D* (*A* and *E*) and the grade 2 carcinoma in Fig. 1*C* (*I*). Neighboring sections of these two tumors were hybridized to UTP[^{35}S]-labeled antisense strand probes for type I transglutaminase (*B*, *F*, and *J*), type IV collagen (*C*, *G*, and *K*), or HPV-16 E6/E7 (*D*, *H*, and *L*). The dark-field micrographs show regions that correspond to the micrographs in *A*, *E*, and *I*. (Bar = 22 μm for *A–D*, 11 μm for *E–H*, and 5.5 μm for *I–L*.) e, Epidermis; ic, inclusion cyst; kp, keratin pearl.

nomas studied. The levels of transgene expression seen in the undifferentiated epidermal cells of these carcinomas approximated that seen in the ocular lens (data not shown), a tissue that is impaired in its development and potentiated for tumor formation in these mice (11).

Cellular Proliferation Correlates with Expression of E6/E7 and Type IV Collagen. Our histological results indicate that hyperproliferation of epidermal cells may characterize the abnormal skin and carcinomas arising in the transgenic mice. We examined the proliferative status of cells in skin tumors using a monoclonal antibody against PCNA, a nuclear protein expressed exclusively in replicating cells (16). PCNA-positive cells were detected in the morphologically undifferentiated epidermal cells surrounding the keratin pearls deep in the dermis of the grade 2 skin tumor in Fig. 1*C* (Fig. 5*B*). These PCNA-positive cells were located in the same regions in which the type IV collagen and E6/E7 probes hybridized in the skin carcinoma (compare Fig. 5*B* to Fig. 4 *K* and *L*, respectively). This localization is consistent with the high expression of E6/E7 contributing to the increased proliferation of the basal or undifferentiated epidermal cells in these neoplastic lesions.

The p53 and Rb Genes are Wild Type in the Skin Tumors of Affected Animals. Given that E6 and E7 may obviate the need for mutational inactivation of p53 and Rb in human cervical cancers, we examined the integrity of p53 and Rb genes in transgenic mouse skin tumors using RNA/PCR-assisted SSCP analysis (representative data are shown in Fig. 6). Migration of all Rb- and p53-specific products from the three tumors examined was identical to the migration of corresponding products generated from nontransgenic mouse skin samples. The conditions used for SSCP analysis permitted detection of a single point mutation in Rb (Fig. 6, lanes p and q) or p53 standards (data not shown). Additionally, these PCR-generated fragments were further examined by DNA sequence analysis; no mutations were found in p53 (between nt 306 and 976) or Rb (between nt 1172 and 2299). These data indicate that the DNA sequence in the commonly mutated regions of the Rb and p53 genes is not mutated in the examined carcinomas.

DISCUSSION

In this study we demonstrate that expression of HPV-16 E6 and E7 in the epidermis of the skin of transgenic mice can lead to a progressive skin disease culminating in a high incidence of squamous cell carcinoma. The histopathological characteristics of this progressive disease are similar to those of HPV-positive cervical disease. This similarity is seen in the preneoplasias, which are characterized by acanthosis and hyperkeratosis, two histopathological properties common to HPV-infected tissue, and in the neoplasias, which are usually squamous cell in origin. A heightened expression of these

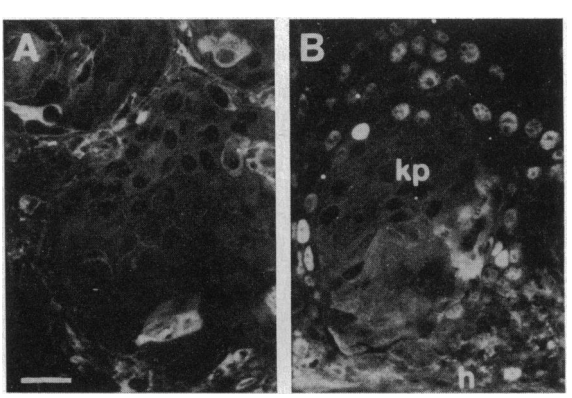

FIG. 5. Analysis of PCNA expression in transgenic skin samples by indirect immunofluorescence. Histological sections (comparable to those in Figs. 3*G* and 4*I*) from a grade 2 carcinoma from a transgenic mouse were immunostained with FITC-conjugated horse anti-mouse IgG secondary antibody only (*A*) and mouse anti-human PCNA monoclonal primary antibody plus secondary antibody (*B*). (Bar = 3 μm.) h, Hypodermis; kp, keratin pearl.

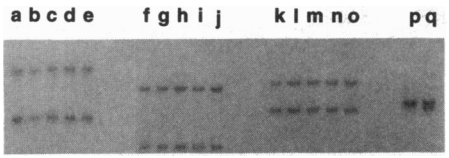

FIG. 6. SSCP analysis of Rb and p53 in skin tumors. Shown is an autoradiograph of RNA/PCR products resolved by gel electrophoresis. RNA samples were from nontransgenic FVB/N skin (lanes a, f, and k), FVB/N embryos (lanes b, g, and l), mouse 554 skin tumor (lanes c, h, and m), mouse 1706 skin tumor (lanes d, i, and n), and mouse 1071 skin tumor (lanes e, j, and o). The primers used for amplification were p53-1 and p53-4 (lanes a–e), Rb-1 and Rb-2 (lanes f–j), and Rb-5a and Rb-6 (lanes k–o). The products in lanes p and q were generated using the primers Rb-3 and Rb-4 and the plasmid templates containing point mutant (amino acid 706, Cys → Phe) or wild-type human Rb cDNAs, respectively. Rb-specific primers used [Rb-1, nt 1152–1172; Rb-2, complementary to nt 1476–1452; Rb-3, nt 1984–2005; Rb-4, complementary to nt 2318–2299; Rb-5a, complementary to nt 1810–1786; Rb-6, nt 1471–1490 (Rb nucleotide assignments as per ref. 17)] were chosen to amplify exons 12–16, 16/17–19, and 20–23. The p53 primers [p53-1, nt 287–306; p53-2, complementary to nt 1006–976; p53-3, nt 568–591; p53-4, complementary to nt 642–619 (p53 nucleotide assignments as per ref. 18)] bracket the commonly mutated sites in the murine p53 gene.

viral genes in undifferentiated epidermal cells is found in the mouse skin tumors and in HPV-positive cervical carcinoma. The parallels between the disease seen in these transgenic mice and cervical carcinoma in patients infected with anogenital papillomaviruses are striking and suggest a role for E6 and E7 in the development of these squamous cell carcinomas.

In this study two of three lines of αAHPV16E6/E7 transgenic mice expressed E6 and E7 ectopically in the skin, and in both cases this expression was associated with adult-onset neoplasia; the cause of this ectopic expression is not known. Although expression of E6 and E7 was detectable in neonatal skin, phenotypically abnormal skin did not develop until 3 months of age or later, and tumors developed only after 6 months of age. A similar latency in tumor formation has been noted in the onset of lens tumors (11) and seminomas (19) in transgenic mice expressing HPV-16 E6 and E7 genes. That viral transgene expression is detectable before onset of disease indicates that additional factors and/or changes in the activity or spatial expression of E6 and E7 proteins within the skin are required for tumor development. Wounding is likely to be a cofactor in the incidence of at least some tumors in these mice. Wounding in skin is known to result in paracrine-based proliferation of epidermal cells (20, 21) and has been found to be a cofactor in the incidence of cancers in other transgenic mice, including animals harboring the bovine papillomavirus type 1 genome (22) and activated cellular Ha-*ras* (23).

By *in situ* hybridization, we found a higher level of expression of E6 and E7 in the skin tumors than in the preneoplastic lesions or in normal transgenic skin. Thus in addition to the likely role of E6 and E7 in the onset of the preneoplastic abnormal skin condition, our results indicate that heightened expression of E6 and E7 may be important for tumor formation. This notion is consistent with the existing hypothesis that the expression of E6 and E7 is not only important in the normal life cycle of papillomaviruses but also in the development of cervical carcinoma (24). In actively infected (preneoplastic) tissue in human patients, expression of E6 and E7 is low in the basal compartment. Likewise, expression of E6 and E7 was low in the basal compartment of the preneoplastic lesions found in the transgenic mice. In contrast, expression of E6 and E7 in cervical carcinoma is selectively high in the expanded basal epidermal cell compartment and correlates with a high percentage of PCNA-positive basal cells (25). This latter pattern of expression of viral and PCNA genes is similar to that observed in the squamous cell carcinomas that developed in our transgenic mice (Figs. 4 and 5). Thus, heightened expression of E6 and E7 specifically in basal or poorly differentiated epithelial cells may be important in the development of carcinoma.

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